EXHIBIT

G

WORKING GROUP MEETING:

Technologies for *in situ* Repair of Single Nucleotide Mutations in Single Gene-Defect Blood Diseases

Monday, September 24, 2001, 8:30 AM- 4 PM
Rockledge II Building
6701 Rockledge Dr. Room 9104
Bethesda, MD
National Institutes of Health

Sponsored by
The National Heart, Lung, and Blood Institute

AGENDA

8:30 am	**Welcome** Greg Evans, Ph.D., Blood Diseases Program, NHLBI
8:40 am	**Efforts to Develop Psoralen-Oligonucleotide Third Strands for Reversing Substitution Mutations** Jacques Fresco, Ph.D., Princeton University
9:10 am	**Targeted Genome Modification Via Triple Helix Formation** Peter Glazer, MD, Ph.D., Yale University
9:40 am	**Gene Repair by Oligonucleotides: Treatment of Skin Disorders** Kyongeun Yoon, Ph.D., Jefferson Medical College
10:10 am	**Break**
10:25 am	**Repair of Single Nucleotide Mutations in Single Gene-Defect Blood Diseases** Cliff Steer, M.D., University of Minnesota
10:55 am	**Sequence-Specific Modification of the Human ß-Globin Gene by Small Fragment Homologous Replacement (SFHR)** Dieter Gruenert, Ph.D., University of Vermont
11:25 am	**Delivery of Oligonucleotides in Cell Culture and *In vivo*.** Ryzard Kole, Ph.D., University of North Carolina, Chapel Hill
11:55 am	**Lunch**
12:55 pm	**Repair of Genetic Instructions by Ribozymes** Bruce Sullenger, Ph.D., Duke University
1:25 pm	**Title to be announced** Don Miller, MD, Ph.D., University of Louisville
1:55 pm	**Break**
2:05 pm	**Group Discussion of Key Topics for Future Focus in Gene Correction Research** Greg Evans, Ph.D., NHLBI
4:00 pm	**ADJOURNMENT**

SEQUENCE-SPECIFIC MODIFICATION OF THE HUMAN ß-GLOBIN GENE BY SMALL FRAGMENT HOMOLOGOUS REPLACEMENT (SFHR)

Goncz KK[1], Prokopishyn N[2], Chow B[2], Davis BR[2], **Gruenert DC**[1]. [1]Human Molecular Genetics Unit, Department of Medicine, University of Vermont, Burlington VT, USA [2]Gene-Cell, Inc., Houston TX, USA

The plethora of hemoglobinopathies with a genetic etiology have been elusive in terms of their response to conventional therapies. The development of genetic therapies has provided new possibilities for treatment of these disorders. While conventional cDNA-based gene therapy has shown some success, there are still numerous limitations in using these strategies as an effective therapeutic modality for inherited diseases. As an alternative to the cDNA-based gene therapy approaches, a number of groups have developed and implemented strategies that directly repair/modify mutant lesions within the genetic DNA. One strategy, small fragment homologous replacement (SFHR), has already been demonstrated to be effective at the simultaneous modification of multiple bases within selectable marker genes (Zeocin) and within the cystic fibrosis transmembrane conductance regulator (CFTR) gene, both *in vitro* and *in vivo*. More recently, SFHR has been applied to site-specific modification of the human ß-globin gene at the genetic locus responsible for sickle cell anemia. Studies were carried out in murine leukemia (MEL) cell lines carrying human chromosome 11 and in isolated human hematopoietic stem/progenitor cells that were $CD34^+$ $CD38^-$ lin^- as well as human bronchial epithelial cells. The cells were transfected, either with DNA lipid complexes (MEL cells) or by direct nuclear microinjection of DNA fragments (human hematopoietic stem/progenitor cells). The fragment used contained the $ß^S$ -globin mutation. After transfection, cells were analyzed either by allele-specific and nonallele-specific DNA polymerase chain reaction (PCR) amplification or RNA reverse transcriptase PCR (RT-PCR) amplification. Nonallele-specific amplification was followed by restriction fragment length polymorphic (RFLP) analysis using the Dde I restriction enzyme. Analysis of the cells indicated that the wild-type $ß^A$-globin had been replaced by the targeting fragment sequences containing the sickle cell mutation (A→T transversion at codon 6). SFHR-mediated modification was observed in the MEL cells as well as the human bronchial epithelial cell line that did not express ß-globin following transfection with a lipid-DNA complex. Microinjection experiments in the human stem/progenitor cells showed stable SFHR-mediated modification. The microinjection experiments were particularly effective for assessing the affect of fragment number on replacement fragment concentration. No replacement was observed at fragment concentrations of < 1000 fragments/fl with an injection volume of 3-5 fl/cell. Replacement was consistently observed at fragment concentrations of 1000 fragments/fl to 5000 fragments/fl. Experiments with human hematopoietic stem/progenitor cells showed that SFHR-mediated modification was stable with continuous growth up to at least 5 weeks. Sequence analysis of a PCR amplicon generated with primers outside the homologous region indicated that, in one experiment up to 70% of the alleles in the cells of the outgrowth population had undergone homologous replacement. In this experiment, 65% of the cells were microinjected and grown for 3 weeks. These studies demonstrate that SFHR-mediated modification can occur in hematopoietic cells at the ß-globin gene locus and that transcription is not necessary for homologous replacement (airway epithelial transfection results). These findings are encouraging for the further development of SFHR as a genetic therapy for hemoglobinopathies. These studies were supported, in part, by NIH grant DK53925 and by funds from Gene-Cell, Inc.

Table 2. PCR and *Xho*I restriction digestion fragments

Primer	Fragment size (bp)
Non-allele-specific DNA	
CF1/CF5[a]	491 (N)
	488 (ΔF)
CF1B/CF6[b]	771 (N)
	768 (ΔF)
Allele-specific DNA	
CF1B/CF7C[b]	392 (N)
*Xho*I	283/109
CF1B/CF8C[b]	389 (ΔF)
*Xho*I	283/106
CF1/CF7C[c]	308 (N)
*Xho*I	199/109
CF1/CF8C[c]	305 (ΔF)
*Xho*I	199/106
CF7B/CF6[b]	414 (N)
CF8B/CF6[b]	411 (ΔF)
Allele-specific RNA	
CF17/CF7C[d]	330 (N)
*Xho*I	221/109
CF17/CF8C[d]	327 (ΔF)
*Xho*I	221/106

Different primer pairs and the resulting fragments following amplification and digestion with *Xho*I. (N), normal genotype; (ΔF), ΔF508 genotype. Conditions for the specific primer pairs were as follows.

[a]Primers 0.4 μM, DNA 50–100 ng, Mg^{2+} 1.5 mM; 94°C for 60 s, denaturation; 55°C for 30 s, annealing; 72°C for 30 s with 4 s/cycle increase, extension; 30 cycles.

[b]Primers 0.5 μM, DNA 50–100 ng, Mg^{2+} 2.0 mM; 95°C for 60 s, denaturation; 59°C for 60 s, annealing; 72°C for 90 s, extension; 35 cycles with 8 min extension on the last cycle.

[c]Primers 0.5 μM, DNA 50–100 ng, Mg^{2+} 2.0 mM; 95°C for 2 min, denaturation; 57°C for 60 s, annealing; 72°C for 90 s, extension; 35 cycles with 8 min extension on the last cycle.

[d]Primers 1.0 μM; DNA 50–100 ng, Mg^{2+} 2.0 mM; 94°C for 30 s, denaturation; 57°C for 30 s, annealing; 72°C for 30 s, extension; 35 cycles with 8 min extension on the last cycle.

Transfection of DNA

Cells were transfected with either the 491 or 488 nt fragments complexed with either the polyamidoamine cascade polymer Starburst dendrimer (13,34) or with cationic lipid (Lipofectamine; Gibco BRL, Grand Island, NY). Dendrimer–DNA complexes were prepared as described previously (13). Complexes with Lipofectamine were made according to the manufacturer's instructions.

Cells were grown in FN/V/BSA-coated T25 tissue culture flasks to ~80% confluence (~10^6 cells). Prior to transfection, the complete medium was aspirated and replaced with 1.5 ml serum-free medium. One of the DNA complexes (4 μg DNA/flask) was then added to the flask and incubated for 5 h at 37°C. The transfection medium was then aspirated and replaced with complete growth medium. Cells were cultured at 37°C after transfection, with daily replacement of medium until the cells were used for DNA and RNA isolation.

DNA and RNA analysis

Genomic DNA and cytoplasmic RNA were isolated and purified from cells (13) 2–3 days following exposure to the fragment. Cellular DNA was analyzed using both non-allele-specific primers CF1B/CF6 (Table 1) and allele-specific primers [e.g. CF1/CF7C or 8C, CF1B/CF7C(8C) or CF7B(8B)/CF6]. Primers CF1B (sense) and CF6 (antisense) are outside the 5′ and 3′ends of the homologous region, respectively, and amplification of DNA from transfected cells gives rise to a mixed population of wild-type and ΔF508 PCR products. Because these primers are also outside the homologous region defined by the template DNA in the pTA vectors, the fragment generated by CF1B/CF6 amplification will not detect unincorporated, randomly integrated fragment or residual template DNA. Allele-specific PCR analysis with primers CF7C and CF8C or CF7B and CF8B (Table 1 and 2) enables differential detection of either wild-type or ΔF508 *CFTR* alleles. CF7B (sense) and CF7C (antisense) are specific for the wt*CFTR* allele, while CF8B (sense) and CF8C (antisense) are specific for the ΔF508 *CFTR* allele. After amplification with CF7C or CF8C, PCR products can also be digested with *Xho*I enzyme as a secondary assessment of SFHR.

Cytoplasmic RNA was analyzed for the presence of ΔF508 CFTR mRNA sequences by reverse transcription (RT) and subsequent PCR amplification (RT–PCR). Briefly, RNA was denatured at 95°C for 2 min, then reverse transcribed. First strand cDNA was amplified using primer CF17 (located in exon 9) and allele-specific primer CF7C (N) or CF8C (ΔF508) (Table 2). PCR products from RT–PCR were also digested with *Xho*I.

PCR amplification product sizes and the conditions for RNA and DNA analyses are as indicated (Table 2). DNA fragments were separated by agarose gel electrophoresis and visualized by ethidium bromide staining.

ACKNOWLEDGEMENTS

We would like to thank Dr Farid Chehab for helpful comments and the lymphocyte control DNA, Dr Frank Szoka for help with the dendrimer complexes, Drs George Caughey, Alessia Colosimo, Aaron Ellison, Y.W.Kan and Hans Schreier for helpful comments and discussions regarding the manuscript, and Linda Escobar and Luz Feeney for expert technical assistance. This work was supported by grants from the National Institutes of Health (DK46002 and DK47766 to D.C.G.; DK09392 to K.K.G.) and a grant from the Cystic Fibrosis Foundation.

REFERENCES

1. Yanez,R.J. and Porter,A.C.G. (1998) Therapeutic gene targeting. *Gene Ther.*, 5, 149–159.
2. Morrow,B. and Kucherlapati,R. (1993) Gene targeting in mammalian cells by homologous recombination. *Curr. Opin. Biotechnol.*, 4, 577–582.
3. Campbell,C.R., Keown,W., Lowe,L., Kirschling,D. and Kucherlapati,R. (1989) Homologous recombination involving small single-stranded oligonucleotides in human cells. *New Biologist*, 1, 223–227.
4. Capecchi,M.R. (1989) The new mouse genetics: altering the genome by gene targeting. *Trends Genet.*, 5, 70–76.
5. Waldman,A.S. (1992) Targeted homologous recombination in mammalian cells. *Crit. Rev. Oncol. Hematol.*, 12, 49–64.

6. Robbins,J. (1993) Gene targeting: the precise manipulation of the mammalian genome. *Circ. Res.*, 73, 3–9.
7. Yamada,G., Sugimura,K. and Stuart,E.T. (1994) Gene-targeting approaches in the study of cellular processes involved in growth or differentiation: advances in analysis of oncogenes, tumor-suppressor genes, cytokine/receptor systems and developmental control genes. *Eur. J. Biochem.*, 226, 739–749.
8. Rubin,E.M. and Barsh,G.S. (1996) Biological insights through genomics: mouse to man. *J. Clin. Invest.*, 98, S7–S11.
9. Wilson,J.M. (1996) Animal models of human disease for gene therapy. *J. Clin. Invest.*, 98, S27–S29.
10. Marth,J.D. (1996) Recent advances in gene mutagenesis by site-directed recombination. *J. Clin. Invest.*, 98, S47–S50.
11. Capecchi,M.R. (1994) Targeted gene replacement. *Sci. Am.*, 270, 52–59.
12. Koller,B.H. and Smithies,O. (1992) Altering gene in animals by gene targeting. *Annu. Rev. Immunol.*, 10, 705–730.
13. Kunzelmann,K., Legendre,J.-Y., Knoell,D.L., Escobar,L.C., Xu,Z. and Gruenert,D.C. (1996) Gene targeting of CFTR DNA in CF epithelial cells. *Gene Ther.*, 3, 859–867.
14. Mulligan,R. (1993) The basic science of gene therapy. *Science*, 260, 926–930.
15. Morgan,R.A. and Anderson,W.F. (1993) Human gene therapy. *Annu. Rev. Biochem.*, 62, 191–217.
16. Capecchi,M.R. (1989) Altering the genome by homologous recombination. *Science*, 224, 1288–1292.
17. Thomas,K.R., Folger,K.R. and Capecchi,M.R. (1986) High frequency targeting of genes to specific sites in the mammalian genome. *Cell*, 44, 419–428.
18. Mansour,S.L. (1990) Gene targeting in murine embryonic stem cells: introduction of specific alterations into the mammalian genome. *Genet. Anal. Tech. Appl.*, 7, 219–227.
19. Goncz,K.K. and Gruenert,D.C. (1998) Site-directed alteration of DNA by small fragment homologous replacement (SFHR). In Gruenert,D.C. and Kmiec,E.B. (eds), *Gene Targeting Vector Protocols*. Humana Press, Totowa, NJ, pp. 165–167.
20. Johnson,L.G., Olsen,J.C., Sarkadi,B., Moore,K.L., Swanstrom,R. and Boucher,R.C. (1992) Efficiency of gene transfer for restoration of normal airway epithelial function in cystic fibrosis. *Nature Genet.*, 2, 21–25.
21. Engelhardt,J.F. and Wilson,J.M. (1992) Gene therapy of cystic fibrosis lung disease. *J. Pharm. Pharmacol.*, 1, 165–167.
22. Kim,H.-S., Popovich,B.W., Shebee,W.R., Shesely,E.G. and Smithies,O. (1991) Problems encountered in detecting a targeted gene by the polymerase chain reaction. *Gene*, 103, 227–233.
23. Meyerhans,A., Vartanian,J.-P. and Wain-Hobson,S. (1990) DNA recombination during PCR. *Nucleic Acids Res.*, 18, 1687–1691.
24. Krishnan,B.R., Kersulyte,D., Brikun,I., Berg,C.M. and Berg,D.E. (1991) Direct and crossover PCR amplification to facilitate Tn*5supF*-based sequencing of lambda phage clones. *Nucleic Acids Res.*, 19, 6177–6182.
25. Schoumacher,R.A., Ram,J., Iannuzzi,M.C., Bradbury,N.A., Wallace,R.W., Hon,C.T., Kelley,D.R., Schmid,S.M., Gelder,F.B., Rado,T.A. and Frizzell,R.A. (1990) A cystic fibrosis pancreatic adenocarcinoma cell line. *Proc. Natl Acad. Sci. USA*, 87, 4012–4016.
26. Kunzelmann,K., Lei,D.C., Eng,K., Escobar,L.C., Koslowsky,T. and Gruenert,D.C. (1995) Epithelial cell specific properties and genetic complementation in a ΔF508 cystic fibrosis nasal polyp cell line. *In Vitro Cell Dev. Biol.*, 31, 617–624.
27. Gruenert,D.C., Basbaum,C.B., Welsh,M.J., Li,M., Finkbeiner,W.E. and Nadel,J.A. (1988) Characterization of human tracheal epithelial cells transformed by an origin-defective simian virus 40. *Proc. Natl Acad. Sci. USA*, 85, 5951–5955.
28. Boggs,S.S. (1990) Targeted gene modification for gene therapy of stem cells. *Int. J. Cell Cloning*, 8, 80–96.
29. Cole-Strauss,A., Yoon,K., Xiang,Y., Byrne,B.C., Rice,M.C., Gryn,J., Holloman,W.K. and Kmiec,E.B. (1996) Correction of the mutation responsible for the sickle cell anemia by an RNA–DNA hybrid oligonucleotide. *Science*, 273, 1386–1389.
30. Kmiec,E.B. (1995) Genetic manipulation in mammalian cells using an RNA/DNA chimeric oligonucleotide. *Adv. Drug Deliv. Res.*, 17, 333–340.
31. Yoon,K., Cole-Strauss,A. and Kmiec,E.B. (1996) Targeted gene correction of episomal DNA in mammalian cells mediated by a chimeric RNA–DNA oligonucleotide. *Proc. Natl Acad. Sci. USA*, 93, 2071–2076.
32. Kren,B.T., Bandyopadhyay,P. and Steer,C. (1998) *In vivo* site-directed mutagenesis of the factor IX gene by chimeric RNA/DNA oligonucleotides. *Nature Med.*, 4, 285–290.
33. Zimmer,A. and Gruss,P. (1989) Production of chimeric mice containing embryonic stem (ES) cells carrying a homeobox Hox 1.1 allele mutated by homologous recombination. *Nature*, 338, 150–153.
34. Haensler,J. and Szoka,F.C. (1993) Polyamidoamine cascade polymers and their use for efficient transfection of cells in culture. *Bioconjugate Chem.*, 4, 85–93.
35. Holmes,A.R., Dohrman,A.F. and Gruenert,D.C. (1997) Cationic lipids improve uptake and stability of small DNA fragments *in vitro*. *Pediatr. Pulmonol.*, 14 (suppl.), 264.
36. Li,S., Brisson,M., He,Y. and Huang,L. (1997) Delivery of a PCR amplified DNA fragment into cells: a model for using synthetic genes for gene therapy. *Gene Ther.*, 4, 449–454.
37. Hunger-Bertling,K., Harrer,P. and Bertling,W. (1990) Short DNA fragments induce site specific recombination in mammalian cells. *Mol. Cell. Biochem.*, 92, 107–116.
38. Kuchertapati,R.S., Spencer,J. and Moore,P.D. (1985) Homologous recombination catalyzed by human cell extracts. *Mol. Cell. Biol.*, 5, 714–720.
39. Cozens,A.L., Yezzi,M.J., Kunzelmann,K., Ohrui,T., Chin,L., Eng,K., Finkbeiner,W.E., Widdicombe,J.H. and Gruenert,D.C. (1994) CFTR expression and chloride secretion in polarized immortal human bronchial epithelial cells. *Am. J. Resp. Cell Mol. Biol.*, 10, 38–47.
40. Gruenert,D.C., Basbaum,C.B. and Widdicombe,J.H. (1990) Long-term culture of normal and cystic fibrosis epithelial cells grown under serum-free conditions. *In Vitro Cell Dev. Biol.*, 26, 411–418.
41. Lechner,J.F. and LaVeck,M.A. (1985) A serum-free method for culturing normal human bronchial epithelial cells at clonal density. *J. Tissue Cult. Methods*, 9, 43–48.
42. Riordan,J.R., Rommens,J.M., Kerem,B.-S., Alon,N., Rozmahel,R., Grzelczak,Z., Zielenski,J., Lok,S., Plavsic,N., Chou,J.-L., Drumm,M.L., Iannuzzi,M.C., Collins,F.S. and Tsui,L.-C. (1989) Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. *Science*, 245, 1066–1073.
43. Rommens,J.M., Iannuzzi,M.C., Kerem,B.-S., Drumm,M.L., Melmer,G., Dean,M., Rozmahel,R., Cole,J.L., Kennedy,D., Hidaka,N., Zsiga,M., Buchwald,M., Riordan,J.R., Tsui,L.-C. and Collins,F.S. (1989) Identification of the cystic fibrosis gene: chromosome walking and jumping. *Science*, 245, 1059–1065.
44. Xu,Z. and Gruenert,D.C. (1996) Human CFTR sequences in regions flanking exon 10: a simple repeat sequence polymorphism in intron 9. *Biochem. Biophys. Res. Commun.*, 219, 140–145.
45. Blanck,A., Glück,B., Wartbichler,R., Bender,S., Pöll,M. and Brandl,A. (1995) Activity of restriction enzymes in a PCR mix. *Biochemica*, 2, 14.
46. Zielenski,J., Rozmahel,R., Bozon,D., Kerem,B., Grzelczak,Z., Riordan,J.R., Rommens,J. and Tsui,L.C. (1991) Genomic DNA sequence of the cystic fibrosis transmembrane conductance regulator (CFTR) gene. *Genomics*, 10, 214–228.